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Fos Activation in the BST Following Juvenile Social Subjugation

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Anxiety and mood disorders such as depression affect more than 60 million adults in the United States alone (Kessler, Chiu, Demler & Walters, 2005). Adverse early-life experiences, such as childhood abuse, are commonly implicated in the development of psychopathology (Tarullo, 2012). Females are disproportionately represented in the number of those affected by these types of disorders (McLean, Asnaani, Litz & Hofmann, 2011). Taken together, this suggests that there may be inherent differences in the way females perceive their environment and process potential threats. As a result, it is imperative that we examine the sexual dimorphism of the brain as it relates to the development of anxiety disorders.

Leuner and Shors (2012) discuss the importance of the stress response as a critical part of adaptive functioning that helps to protect the body from immediate harm. A fear response can generate a memory that later facilitates an anxious state that allows for proper avoidance of threat. In healthy individuals, after a threat has passed, arousal levels return to baseline. However, fears can become generalized to non-threatening stimuli or contexts and lead to a disordered stress response. The mechanism by which fears become generalized is likely sexually dimorphic and has yet to be elucidated.

Several structures in the brain are implicated in facilitating the stress response. The amygdala is the first structure to alert the body to a potential threat in the environment. Among its many functions, the amygdala mediates emotional response and emotional memory formation (Staniloiu & Markowitsch, 2012). The central nucleus of the amygdala (CeA) responds to physical stress (Dayas et al., 2001) whereas the medial amygdala mediates sex-specific social behavior (Thierry et al., 2010). Its sub-nuclei effect physiological arousal through projections to

several other structures, including the hypothalamus and the bed nucleus of the stria terminalis (BST) (Swanson & Petrovich, 1998).

The hypothalamus is part of a larger neural circuit known as the Hypothalamic-Pituitary-Adrenal Axis (HPA), which controls the neuroendocrine stress response. Specifically, the HPA mediates hormone response and this dysregulation is implicated in the development of anxiety and mood disorders (Schutter, 2012). A stressor induces the release of Corticotropin Releasing Hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus (Frodl & O'Keane, 2012). CRH is carried to the anterior pituitary where it triggers the release of adrenocorticotropin (ACTH) that in turn causes the release of glucocorticoids such as cortisol (corticosterone in rodents) (Frodl & O'Keane, 2012). The end result is the body prepared for "fight or flight". Too much circulating cortisol stops the release of both CRH and ACTH and returns the body to its baseline arousal (Frodl & O'Keane, 2012).

The BST occupies the forebrain and is located in both hemispheres, surrounding the anterior commissure (Swanson, 1998). It receives innervations from the MeApd, CeA, basolateral nucleus of the amygdala and has come to be classified as part of the "extended amygdala" (Swanson & Petrovich, 1998).

Herman and colleagues (1994) identified the BST as being involved in the stress response. They found that it plays a critical role in the activation of the HPA axis through its projections to the paraventricular nucleus and subsequent influence on its expression of CRH. They demonstrated that large lesions of the principal nucleus of the BST (BSTpr) resulted in decreased CRH mRNA expression in the PVN and thus, a decreased stress response. Additionally, they revealed that the BST also provides inhibitory inputs to the PVN to influence CRH expression. It has also been implicated in behavioral responses such as freezing and startle

in relation to innate fears. Several studies have investigated the role of the BST in the innate fear of mice to the odor of cat urine (Luyten, van Kuyck, Vansteenwegen & Nuttin, 2011). Of critical importance to this line of research is the fact that the BST is a sexually dimorphic structure (Polston, Gu & Simerly, 2004) that appears to mediate both immediate and sustained fear responses characteristic of anxiety (Davis, Walker, Miles & Grillon, 2010).

Adverse early life experiences can affect the developing brain in ways that over-sensitize it to stress (Andersen & Teicher, 2009). Increased stress during developmental "critical periods" can have long-lasting negative effects on brain function (Baram et al., 2012). The effects of chronic stress have been shown to increase the incidence of psychopathologies like anxiety and depression (Tarullo, 2012), decrease the volume of the hippocampus, impairing memory, and can handicap the immune system (Frodl & O'Keane, 2012,) along with other deleterious effects.

The current study is an extension of previous work done in this lab that examined the effects of juvenile social subjugation (JSS) on adult behavior in the rat (Weathington & Cooke, 2011). Results indicated that although both juvenile males and females experienced JSS, females exhibited significantly more anxiety and depression-like behaviors than their male counterparts as adults. This finding prompted interest in neural structures that may process juvenile stress in a sex-specific manner.

Many studies have examined sex differences in the rat brain after the activational effects of pubertal hormones have taken place and further differentiated the male and female brain. Fewer studies have investigated sex differences in the juvenile brain, how adverse experiences might be processed, and implications for altered developmental trajectories. Investigation of the prepubertal brain, as it has only been exposed to the organizational effects of hormones in utero,

allows us to observe the effects of stress without the added variable of circulating gonadal hormones.

Following similar procedures as Weathington and Cooke (2011), a rat model of juvenile social subjugation was used to elicit a social-stress response. One additional control group was added to eliminate potential confounds. A Benign Control (BC) was used to differentiate the stress of interaction with an unfamiliar conspecific and the stress of an aggressive adult male. A Handled Control (HC) Condition was included, as previously, to rule out the influence of handling stress when subjects are moved between cages.

Both the anterior and posterior regions of the BST were separately analyzed in the present study. From the anterior division, the anterodorsal (BSTad) and anterolateral (BSTal) nuclei were chosen and from the posterior division, the principle nucleus (BSTpr) was selected (Figure 1.). The current study seeks to identify sex differences in total neuron number and neuronal activation in the bed nucleus of the stria terminalis. Because the BSTpr has been shown to be sexually dimorphic in adult animals, it was hypothesized that the BSTpr would exhibit a sex difference in total neuron number. Additionally, it was predicted that the posterior BST would exhibit significantly more Fos activation as a result of innervations from the CeA, MeA and BLA, which are all involved in the stress response. Finally, the JSS condition should elicit higher Fos activation than the Benign Control or Handled Control conditions.

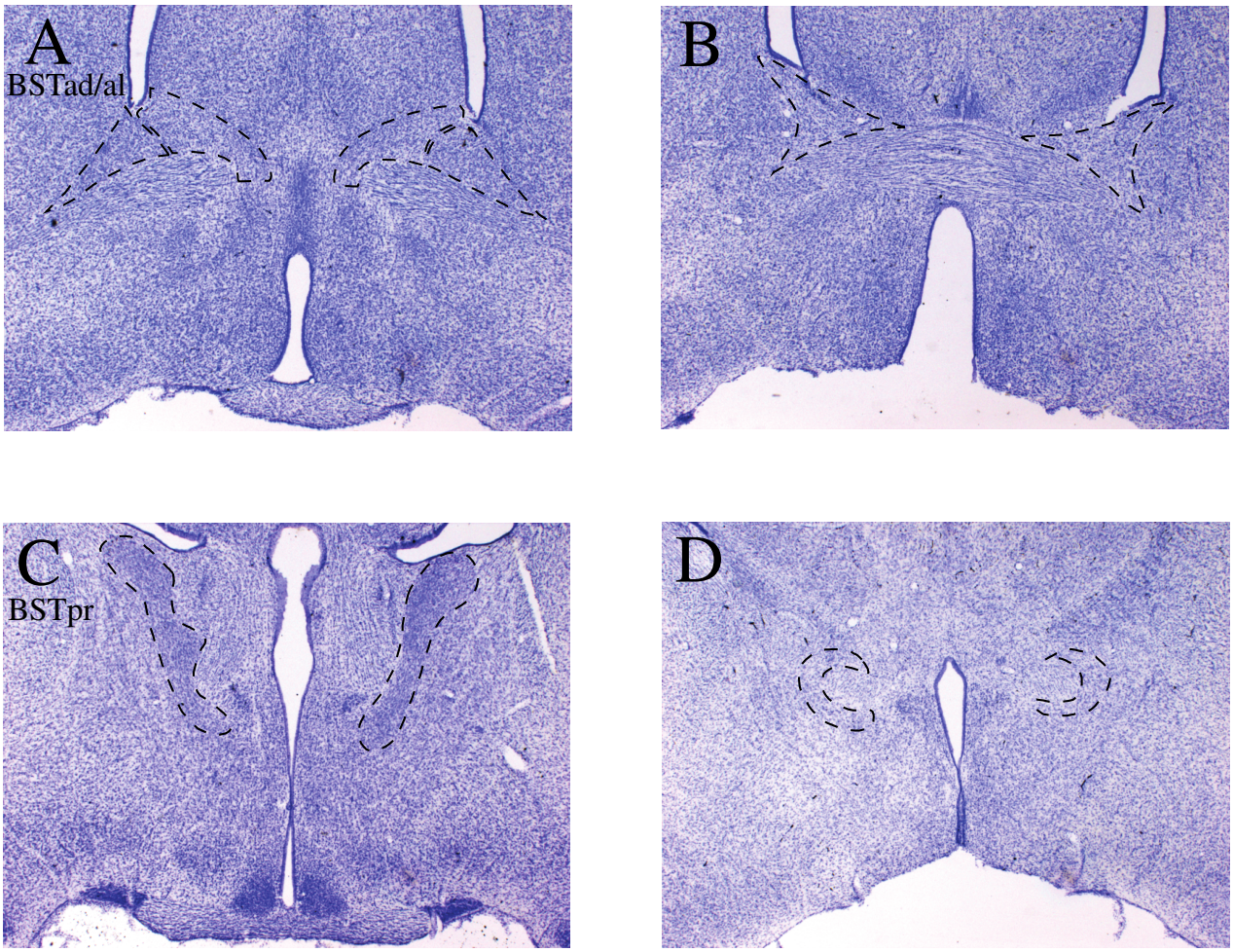

Figure 1. Photomicrographs of four, representative rostro- caudal levels of the BST.

Method and Materials

All methods were approved by the Georgia State University Animal Care and Use Committee and conducted in accordance with the *N.I.H. Guide for the Use of Laboratory Animals.* Subjects were housed in a vivarium on a reversed light cycle at 22˚C with ad libitum access to food and water. Efforts were made to reduce the number of animals used and minimize their suffering.

Screening Aggressive and Benign Residents

Adult male Long Evans rats (N=15) from Charles River arrived at postnatal day 60 (P60) to be screened for reliability of aggression. A male or female juvenile rat (P28) was placed into

the home cage of an adult male for 10 minutes. During this time, the frequency of kicks, pins, and dominance postures by the adult was noted. Kicks were defined as the use of the back paws to kick the juvenile intruder. Pins were the immobilization of the juvenile in a supine position, whereas dominance postures immobilized the juvenile in a prone position with the use of the front paws. The seven most aggressive males from the cohort were used as the Resident Aggressors (RA) in the JSS condition.

Resident Aggressors were housed in 53 x 29 x 20 cm cages that were equipped to hold a clear partition. In order to maximize aggressiveness, cage changes were reduced to once per week and RAs were housed with a receptive female. Each female cage mate was ovariectomized and received bi-weekly injections of estradiol (0.1 mg) followed by progesterone (0.5 mg) 24 hours later.

A separate cohort of males (N=15) was used to identify benign controls. A juvenile was placed into the home cage of the adult male for 10 minutes and aggressive acts were noted. Only adults that exhibited no aggressive behavior towards the juvenile met the criteria for a benign male. Of the original cohort, five rats were selected and housed in 45 x 23 x 20 cm cages.

Juvenile Social Subjugation Procedure

P21 male and female rats (N=36) arrived three weeks after the completed screening for aggressive and benign males. Aside from feeding and cage changes, juveniles were left undisturbed for six days in 45 x 23 x 20 cm cages following their arrival. Rats were then randomly assigned to the Handled Control (HC), Benign Control (BC) or Juvenile Social Subjugation (JSS) condition. Each condition was composed of six males and six females between P28 and P33.

The JSS condition was modeled after the resident-intruder paradigm used by Miczek (1979). First, the RA's female cage mate was removed and a juvenile was placed behind the clear partition of the cage. After five minutes, the clear partition was removed. Kicks, pins and dominance postures were noted. At the expiration of 10 minutes, or after 10 aggressive acts by the RA, the partition was replaced and the juvenile was put behind it for five additional minutes before being returned to its home cage. Physical interaction during the JSS condition was stopped after 10 minutes or 10 aggressive acts to ensure similar experiences between subjects. An animal was excluded from the study if there was any observed sexual behavior, or if the juvenile received fewer than 10 aggressive acts in the allotted time. Additionally, RAs were only used a maximum of two times.

The Benign Control Condition followed the same procedure except that the juvenile was placed with a Benign male. The juvenile was excluded from the study if any sexual behavior was observed or if the juvenile experienced an aggressive act. Benign rats were also used a maximum of two times.

The Handled Control Condition followed the same procedures except that the juvenile was placed into a clean, empty cage with fresh bedding. This condition was intended to rule out any stress attributable to the subjects' transfer from one cage to another by the experimenter.

Tissue Collection

Subjects were sacrificed one hour after being returned to their home cage. They were anesthetized with pentobarbital and perfused with 4% paraformaldehyde (200mL) in 0.1M phosphate buffer (pH 7.2). Brains were then dissected and blocked at the optic chiasm and the cerebellum. Brains were allowed to post-fix overnight at 4˚C in perfusate solution. The following day they were placed in 30% sucrose for 72 hours. Scores were placed on the right hemisphere to

ensure correct tissue mounting for analysis of laterality. Brains were sliced at 40µm using a freezing microtome and the serial sectioning was completed in four-series. Sliced tissue was stored at -20˚C in cryoprotectant in 12-well plates until analysis.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted to visualize neuronal activation in the BST. The process of IHC capitalizes on the biological function of antibodies to attach to antigens. In this case, the antigen of interest is the Fos protein. The presence of Fos in the nucleus of a neuron is indicative of the activity-inducible immediate early gene *c-Fos*, which is expressed in response to intense neural activity. The expression of *c-Fos* promotes the transcription of proteins that ultimately cause long-term changes in neural function, such as learning. The binding of antibodies to the Fos protein is then visualized through the use of a peroxidase which causes a black precipitate to form and can be viewed using a microscope. Figure 1 depicts the appearance of a Fos-expressing neuron at 100 X magnification.

To begin the process of visualizing Fos, brain tissue was rinsed (10 x 5 minutes) in 0.1 M phosphate buffered saline (PBS) to remove all traces of cryoprotectant. Tissue was then incubated for 48 hours at 4°C in rabbit anti-c-Fos IgG antibody (Santa Cruz Biotechnology) which was diluted to a ratio of 1:10,000 in PBS containing 0.4% Triton X-100 (PBTX). Triton X-100 is a detergent used to permeabilize cellular membranes, which enables the antibody to attach to the Fos protein within in the nucleus. Tissue was washed again in PBS (10 x 5 minutes) and incubated for 1 hour at room temperature in biotinylated goat anti-rabbit IgG secondary antibody (Jackson Immunoresearch Inc.), diluted to 1:600 in PBTX.

Following the same wash procedure with PBS, tissue was incubated in an avidin-biotin solution (ABC peroxidase ‘Elite’ kit, Vector Laboratories) of 45mL PBTX with 20 µl Reagent A

and 20 µl Reagent B for 1 hour at room temperature. Sections were washed in PBS (5 x 5 minutes) and then washed in a 0.175 M sodium acetate solution (3 x 5 minutes). The tissue was incubated in 0.175 M sodium acetate solution containing 0.3% H_2O_2, 2.5% nickel ammonium sulfate, and 0.02% 3, 3, diaminobenzidine (Sigma, cat. # 32750), for 15 min at room temperature. Finally, sections were washed in sodium acetate (3 x 5 minutes), PBS (5 x 5 minutes), and stored in 0.1 M phosphate buffer at 4°C.

Tissue Mounting and Staining

Tissue was mounted rostro-caudally onto gelatin coated slides that had been coded to ensure unbiased analysis. Slides were allowed to dry overnight at 37°C. The following day, tissue was counterstained with cresyl violet. After leaving the dye, slides were put through baths of increasing alcohol concentration to dehydrate the tissue. Slides were then individually placed into xylene to clear the tissue of residual alcohol and fats, and were coverslipped using permount.

Stereology

StereoInvestigator (MBF Biosciences, Williston, VT, USA) software was used to analyze the percentage of Fos activation and overall number of neurons in the BST. Stereology was conducted using the optical fractionator method that calculates three-dimensional information about a given nucleus using the tissue sections obtained from the 1:4 sectioning procedure. Contours are drawn around the nucleus of interest on each tissue section. The program then provides systematic, randomly- selected sampling sites from which to count Fos-positive and Fos-negative neurons. The thickness of each section is taken into account and several statistical analyses are performed.

Contours were traced using a 2.5x objective with the use of the Swanson (1998) rat brain atlas. Counting frame height was 6 μm with 2 μm guard zones on either side. Neurons were counted using a 100x oil-immersion lens and were only included if they were within the confines of the counting frame or if they were touching one of the inclusion lines of the frame. As shown in Figure 2, Fos-positive neurons were distinguished by a black precipitate in the nucleus whereas Fos-negative neurons were only distinguishable by their cresyl violet staining. Glial cells were easily identified as having a rougher-looking surface texture, less bulbous shape and were generally smaller in size. The anterior BST was counted using a grid size of 150x150; however, for the posterior BST, a grid size of 90x90 was chosen. Parameters were selected to achieve total cell counts of 150-200 per region to improve stereological precision.

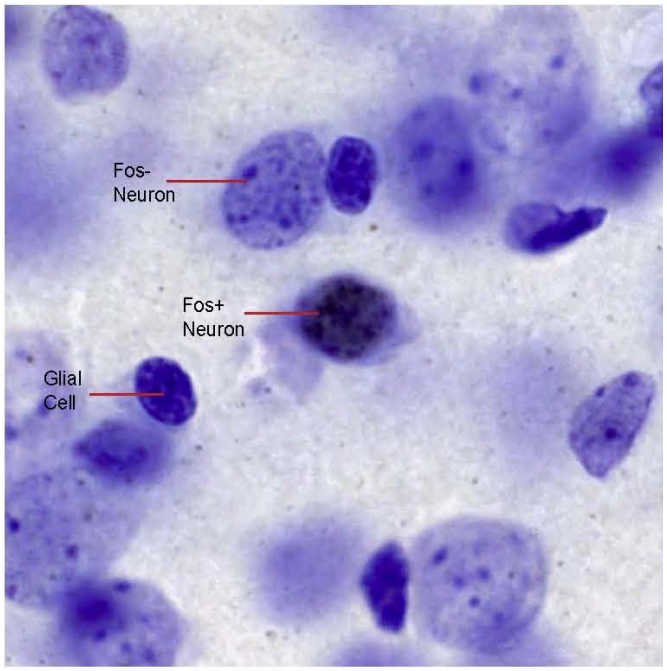

Figure 2. Appearance of a Fos-expressing neuron in comparison to a Fos-negative neuron and glial cell.

Statistical Analysis

Animals were excluded from fraction of Fos and total cell analysis if fewer than150 cells per region were counted. This was often the result of poor tissue quality or missing tissue.

Animals were excluded from total neuron analysis if the number of contours drawn was more than two standard deviations from the mean. Excluding highly counted brains from the fraction of Fos analysis was not necessary due to increased stereological precision with increased sampling.

To investigate effects of hemispheric laterality, analysis of total cell number and fraction Fos-positive cells were first investigated with a two-way repeated measures analysis of variance (ANOVA) with cerebral hemisphere as the within-subjects variable, and sex and condition as the between subjects variables. If no effect of laterality was detected, data were collapsed across hemispheres and analyzed with a repeated measures two-way ANOVA where anterior/posterior region was the within-subjects variable and sex and condition were the between-subjects variables. Upon observing statistically significant main effects or interactions, post hoc tests were performed using student's t-tests. Significance for all analyses was set at $\alpha = p < 0.05$.

Results

Total cell number

Because no effect of hemispheric laterality was observed in the anterior BST (data not shown), total cell number was collapsed and analyzed across cerebral hemispheres. No sex difference in the total number of neurons was observed in the anterior BST ($p = 0.5$; Figure 3). Unlike the anterior BST, sex significantly interacted with hemisphere ($F_{1,29} = 7.89$, $p = 0.009$) in the posterior BST. Post hoc comparisons revealed no effect of sex on total neuron number in the left posterior BST ($p = 0.82$), however; sex significantly affected total cell number in the right posterior BST ($p = 0.01$; Figure 4), with males having more neurons than females.

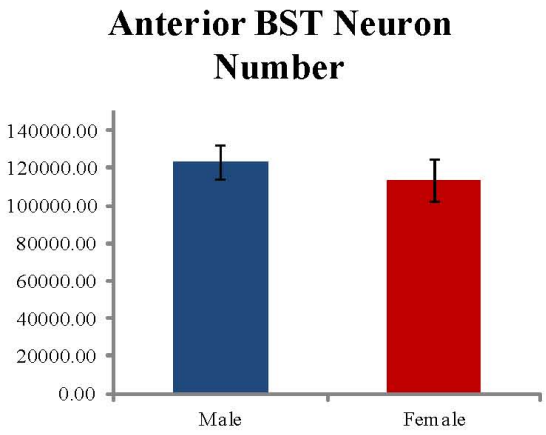

Figure 3. No sex difference between males and females in anterior BST neuron number.

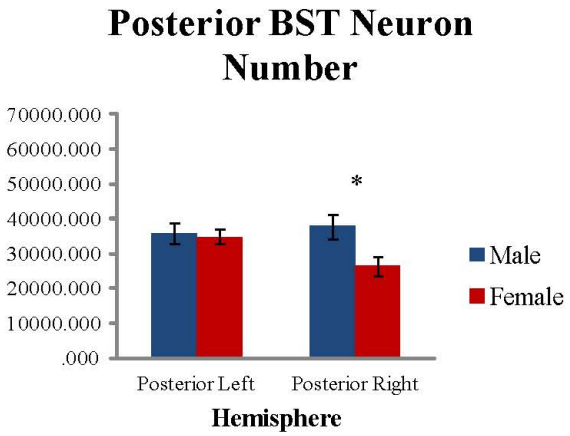

Figure 4. An interaction between sex and hemisphere in the right posterior BST.

Fraction of Fos-Positive Neurons

There was an overall, statistically significant effect of rostro-caudal position on Fos activation. The posterior BST displayed more Fos-positive neurons than the anterior BST, regardless of sex or condition ($p = 0.04$; Figure 5).

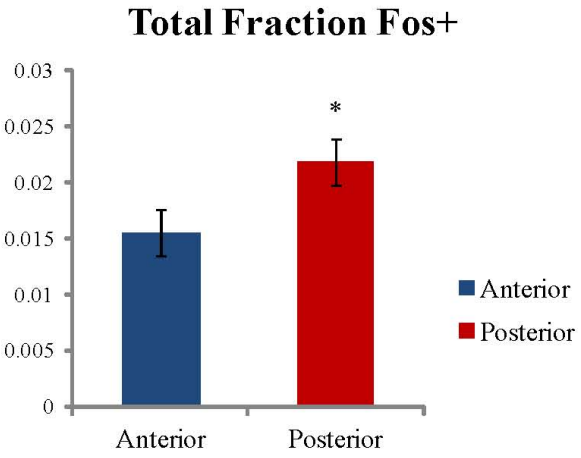

Figure 5. Significantly more Fos activation in the posterior BST.

Anterior BST. No main effect or interaction of hemispheric laterality was detected on fraction of Fos positive neurons in the anterior BST (data not shown). Therefore data were pooled across cerebral hemispheres. Furthermore, neither sex ($p = 0.86$), condition ($p = 0.24$),

nor a sex-by-condition interaction (p = 0.65) significantly affected Fos expression in the anterior BST (Figure 6).

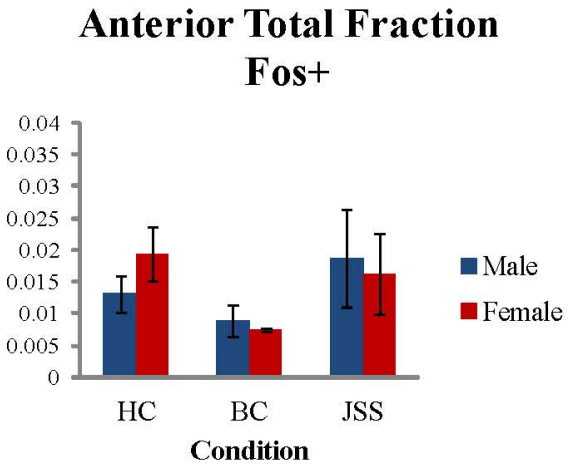

Figure 6. No main effect of condition, sex or interaction on fraction Fos- positive neurons in the anterior BST.

Posterior BST. Hemispheric laterality did not have a main effect or interact with fraction of Fos-positive cells in the posterior BST (data not shown). As a result, data were pooled across cerebral hemispheres. No main effect of sex (p = 0.42) or a sex-by-condition interaction (p = 0.34) on posterior BST Fos activity was observed (Figure 6). There was a strong trend toward a main effect of condition (p = 0.05) on fraction of Fos-positive posterior BST neurons (Figure 7).

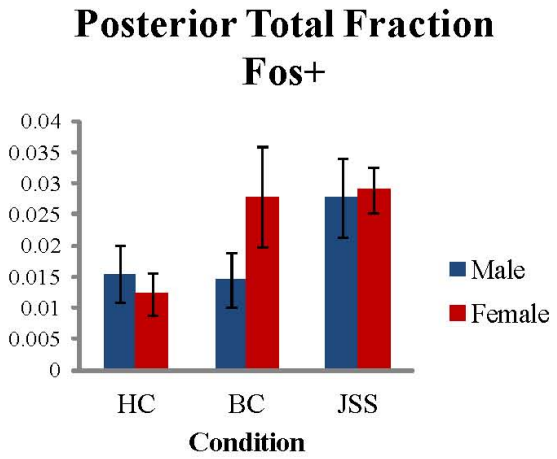

Figure 7. No main effect of sex, condition ot interaction on fraction Fos-postive neurons in the posterior BST.

Discussion

Due to the disproportionately high incidence of anxiety and mood- related disorders in females, the current study investigated effects of stress on the sexually dimorphic bed nucleus of the stria terminalis. While the BST is known to mediate sex-specific behavioral responses to stress in adulthood, the effects of juvenile stress on this region have not been examined. The effect of juvenile social stress on the BST was investigated by subjecting juvenile rats to social subjugation by an adult male, interaction with a benign adult male, or a handled control condition. One hour after the experience, rats were sacrificed and immunohistochemistry was conducted to visualize Fos protein indicative of neuronal activation. It was hypothesized that a structural asymmetry of neuron number as well as functional asymmetry of Fos activation would be observed; specifically that the posterior BST would display an effect of laterality, and that the posterior BST would exhibit more Fos-positive activation and that the JSS condition would elicit significantly more Fos activation than the other two conditions.

Sexual Dimorphism in Neuron Number in the Posterior BST

The BST, as well as other structures that innervate the BST, such as the medial amygdala (Cooke & Woolley, 2005), are known to be sexually dimorphic in adult rats (Polston, Gu & Simerly, 2004). Because many sexually dimorphic structures exhibit hemispheric asymmetry or laterality (Morris, Jordan & Breedlove, 2008; Duchesne, Dufresne & Sullivan, 2008), we investigated whether the BST may be lateralized. No effect of hemispheric laterality on the number of neurons was observed in the anterior BST, nor was there a sex difference in the total number of neurons. However, there was a significant interaction between sex and laterality in the posterior BST where males had a greater number of neurons than females in the right, but not the left hemisphere. This is an interesting finding that mirrors the laterality pattern found in the

posterior dorsal medial amygdala with which the BSTpr is highly connected. Cooke, Stokas and Woolley (2007) found that the right MeApd has more neurons in males than in females, whereas no sex difference in neuron number occurs in the left MeApd.

Functional Dimorphism in the Posterior but Not the Anterior BST

As predicted, the posterior BST reacted with more Fos activation than the anterior, regardless of sex or condition. This prediction was based upon evidence that the posterior BST is heavily innervated by projections from the central and medial nuclei of the amygdala, which are involved in mediating physical stressors and social interaction, respectively (Dayas et al., 2001). This anterior-posterior difference seems to indicate that the anterior region may not process social threat to the same extent as the posterior BST.

No main effect of laterality or interaction of hemispheric laterality with the other independent variables was detected on the fraction of Fos-positive neurons in the anterior BST. Additionally, there were no significant main effects of sex, condition, or a sex-by-condition interaction on Fos expression in the anterior BST.

Similarly, hemispheric laterality did not have a main effect or interact with fraction of Fos-positive cells in the posterior BST. While neither a main effect of sex, or a sex-by-condition interaction on posterior BST Fos activity was observed, a main effect of condition neared statistical significance ($p = 0.05$). After closely inspecting the data, it was noted that the female BC group had a large standard error that appeared to be driven by one animal with an unusually low amount of Fos activation. This anomalous outlier is likely obscuring the predicted effect of condition on posterior Fos activation. As with many animal studies, the limited sample size obtained for this study may be a limiting factor in observing statistical significance.

In the posterior BST, males and females responded similarly to the JSS condition with 33% more Fos activation than the Handled Control animals. However, response differed between males and females in the Benign Control condition. Females exhibited a similar level of response to the benign male as they did to the aggressive male. This suggests that the juvenile females reacted to the presence of an adult male, irrespective of its actions, while the juvenile males reacted to the aggressive behavior encountered in the JSS condition.

This interpretation could be further investigated with the use of adult females as stimulus animals in the JSS and benign control conditions. This would clarify whether the juvenile females were responding to the adult's sex specifically, as opposed to the type of social interaction. Additionally, if this is the case, further studies could then be conducted to determine whether this difference is due to the organizational effect of hormones. If juvenile female brains were masculinized by neonatal androgen treatments, perhaps they might display the same pattern of results as males. It would also be interesting to determine the extent to which the function of the BST changes as a result of exposure to pubertal hormones.

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